

Correlative genital tract morphology and plasma progesterone levels during the ovarian cycle in Corn mice (*Calomys musculinus*)¹

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Abstract

Described the correlation between genital tract morphology and plasma progesterone in laboratory-reared corn mice (*Calomys musculinus*). Morphological traits of the ovary, uterus and vagina were used to define stages of the estrous cycle and of some anovulatory states. These findings were correlated with changes in the vaginal smear and in plasma progesterone (as measured by radioimmunoassay). Estrous cyclicity and ovulation occurred in females caged without males. Plasma progesterone during the estrous cycle showed both a periovulatory and a postovulatory peak. Changes in the vaginal smear correlated poorly with ovarian changes, except the formation of new corpora lutea, that correlated with a sudden disappearance of cornified cells from the smear. The most frequent cycle length was 5 days. Finally, two histologically distinct anovulatory conditions were found associated with basal progesterone levels. The ovarian cycle of this species is provisionally classified as type III of CONAWAY (1971), (spontaneous ovulation, induced luteal phase).

Introduction

Calomys musculinus, the corn mouse, is probably the most widespread species of field mice in Argentina. It is found from near Esquel (43° South latitude) to the tropical boundaries with Bolivia and Paraguay, and from the Andes (2230 meters altitude at Maymará, the type locality, province of Jujuy) to sea level in the province of Buenos Aires. In the humid and temperate Pampa's grasslands this species acts as reservoir of Junín virus, the etiologic agent of Argentine hemorrhagic fever (SABATTINI et al. 1977; WEISSENBACHER and DAMONTE 1983).

Reproductive mechanisms of the corn mouse may also be interesting in the context of the evolution of muroid rodents, because South American cricetids are a monophyletic group (Subfamily Sigmodontinae; REIG 1980) that probably differentiated from North American cricetids in the early Miocene (REIG 1982), some 22 million years ago. The murids (laboratory rats and mice included), whose reproductive biology is, by far, the best known, only emerged as a distinct group some 15 million years ago (WALKER 1964).

The corn mouse breeds mainly in the spring and summer, but can also reproduce all year round, particularly when winters are not too severe (MILLS et al. 1992). In the laboratory, it has a gestation period of 21 days, a mean litter size of 4.4 to 7.6 young and a postpartum estrus (DE VILLAFANE 1981; HODARA et al. 1984).

The present paper describes the ovarian, uterine and vaginal changes occurring during the estrous cycle of this species, and in two distinct anovulatory conditions that were found. Also, plasma progesterone levels were measured in both cycling and anovulating females, and in males.

¹ This work is dedicated to Professor JOHN W. EVERETT on his 85. birthday.

Material and methods

Animals

Colony bred animals were studied after 7–8 weeks of age (fertile copulations can occur as early as the 4th week; DE VILLAFANE 1981). The colony originated in 1983 from wild mice captured in two localities (La Pega and Nacuñán) of the province of Mendoza. Animals from the two localities have been bred as separate lines ever since. However, there were no apparent differences between lines, and all the groups presented here were composed of females from both. The females were kept in groups of 4–8, in 12 × 20 × 30 cm cages, without males within the cage, under 14 h light/10 h dark, and at 24 °C, and they had free access to a balanced diet and water.

Vaginal smears

Vaginal smears were obtained daily (for periods of 14 to 54 days) in most females, by vaginal lavage with a small pipette. The smears were dried in an oven, fixed in ethyl ether and absolute ethanol (1:1), and stained by the method of PAPANICOLAOU (1954), with minor modifications. In some cases, the material for smearing was not taken, because the vaginal orifice was extremely narrow or closed.

Sacrifices and plasma sampling for progesterone determination

All sacrifices were made in the evening. One hundred and sixty one females were sacrificed under ether to establish the correlation between ovarian histology and the other features of the genital tract. Vaginal smears were taken from 114 of these females, before sacrificing them at selected stages of the vaginal smear. The remaining 47, from which smears were not taken, were sacrificed on random days. The uterine horns were inspected for the presence of intraluminal fluid, before the genital tract was removed. The ovaries and uteri were trimmed of surrounding tissues, and weighed. Ovaries and vaginas were fixed in Stieve's fluid and embedded in paraffin. Tissue sections were stained with haematoxylin and eosin. The ovaries were sectioned serially.

Additionally, 80 females and 10 males were sacrificed by decapitation and trunk blood was collected in heparinized tubes for progesterone determinations. Females were sacrificed at selected stages of the vaginal smear, and were autopsied as described in the above paragraph. Plasma was separated by centrifugation and kept frozen until the day the radioimmunoassay was performed as previously described (BUSSMANN and DEIS 1979; CASTRO-VAZQUEZ and CARRENO 1981).

Ovarian follicles

The largest follicle with no signs of atresia, was classified as either type 5b, 6, 7 or 8, according to PEDERSEN and PETERS (1968): 5b-largest preantral follicles; 6-growing follicles with antral lacunae; 7-growing follicles with a single antral cavity and a well-defined cumulus oophorus; and 8-Graafian follicles with a definite cumulus stalk.

Corpora lutea (CL)

They were classified in four classes, that seem to represent the sequence of corpus luteum (CL) formation and regression, and that were defined as follows: Ia – approximately spherical CLs, with or without a central cavity (the collapsed 'channel' through which the ovum was apparently shed, could still be recognized in CLs without a cavity); the cavities were sometimes filled with blood cells and/or fibroblasts; the luteal cells were large and lightly acidophilic, with clear nuclei and distinct nucleoli; frequently both hollow and solid CLs were seen in the same animal and, therefore, were considered as a single class; Ib – spherical CLs with no cavities or 'channels', and with luteal cells as in Ia; II – CLs that were frequently deformed by adjacent structures and showed an increased proportion of fibroblasts; most luteal cells were smaller than in Ia or Ib, and their nuclei were usually smaller and/or darker, and had absent or un conspicuous nucleoli; and III – small and distorted CLs with much connective tissue surrounding groups of small luteal cells with lightly stained cytoplasm.

Statistics

Multi-group comparisons between means (ovarian and uterine weights) were made by analysis of variance, using the Tukey test for post-hoc analysis. Multi-group comparisons of the incidence of vaginal cornification, uterine fluid or old corpora lutea were made with the chi-square test (2-tailed)

for k-samples. Post-hoc analysis between two groups was made either with the chi-square test or with the Fisher's exact probability test, as recommended in STEGEL (1956). Significance level was fixed at $P < 0.05$.

Results

I - Ovarian stages and their associated uterine and vaginal changes

Females that exhibited ovarian signs of recent or impending ovulation, were classified as proestrous, estrous, metestrous or diestrous, according to criteria defined below. However, near 50 % of females were in one of two anovulatory conditions. The main features of these ovulatory and anovulatory classes were as follows (see also Tables 1 and 2).

Table 1. Age and body weight, and histological features of the ovary, in female Corn mice in different ovarian stages

| Ovarian stage | Number of cases | Age, days (a) | Body weight, g (b) | Type of the largest follicle | Type of the most recent corpus luteum |
|----------------|-----------------|---------------|--------------------|------------------------------|---------------------------------------|
| Proestrus | 14 | 163 ± 11 | 19.3 ± 0.8 | 7 | none, II or III |
| Estrus | 21 | 130 ± 15 | 17.1 ± 0.8 | 8, or recently ruptured ones | none, II or III |
| Metestrus | 26 | 163 ± 10 | 18.8 ± 0.7 | 5b-6 | Ia |
| Diestrus | 23 | 163 ± 12 | 19.3 ± 0.8 | 5b-6 | Ib or II |
| Atretic cycles | 13 | 140 ± 17 | 16.6 ± 0.7 | 5b-6 | none, II or III |
| Anestrus | 64 | 146 ± 5 | 16.8 ± 0.4 | 5b-6 | none or III |

(a) Mean ± S.E.M. No significant differences (Tukey test); (b) Mean ± S.E.M. 'Anestrus' differed significantly from all groups except 'Estrus' and 'Atretic cycles'. 'Atretic cycles' differed significantly from 'Proestrus' and 'Diestrus' ($p < 0.05$; Tukey test).

Table 2. Other ovarian, uterine and vaginal features in female Corn mice in different ovarian stages (a)

| Ovarian stage | Ovarian weight (b) mg | Old corpora lutea (c) % | Uterine weight (b) mg | Distended uteri (d) % | Open vaginas (e) % | Cornified vaginas (f) % |
|----------------|-----------------------|-------------------------|-----------------------|-----------------------|--------------------|-------------------------|
| Proestrus | 4.9 ± 0.4 | 85.7 | 60.4 ± 11.3 | 42.9 | 71.4 | 58.3 (12) |
| Estrus | 4.5 ± 0.3 | 85.7 | 56.5 ± 4.6 | 90.5 | 100.0 | 94.7 (19) |
| Metestrus | 4.4 ± 0.2 | 65.4 | 50.0 ± 5.2 | 19.2 | 96.0 (25) | 0 (24) |
| Diestrus | 4.5 ± 0.3 | 73.9 | 43.9 ± 2.5 | 13.0 | 87.0 | 0 |
| Atretic cycles | 3.0 ± 0.4 | 69.2 | 26.9 ± 4.7 | 15.4 | 81.8 (11) | 30.8 |
| Anestrus | 2.2 ± 0.1 | 10.9 | 11.9 ± 0.6 | 0 | 37.5 | 19.3 (57) |

(a) Values which are not percentages are expressed as mean ± S.E.M. Unless otherwise indicated in parentheses, the number of cases in each group is the same as in Table 1; (b) 'Anestrus' and 'Atretic cycles' differed significantly between them and from all other groups ($P < 0.05$; Tukey test); (c) % of animals with type II and/or type III corpora lutea. Only 'Anestrus' differed significantly from all other groups ($P < 0.05$; Chi-square test); (d) 'Anestrus' and 'Estrus' differed significantly between them and from all other groups ($P < 0.05$; Chi-square test, followed by Chi-square or Fisher's test as 'post hoc' analysis); (e) 'Anestrus' differed significantly from all other groups. Also, 'Proestrus' differed significantly from 'Estrus' and 'Metestrus' ($P < 0.05$; Chi-square test, followed by Chi-square or Fisher's test as 'post hoc' analysis); (f) 'Anestrus' differed significantly from all groups except 'Atretic cycles'. Also, 'Atretic cycles' differed significantly from 'Estrus', 'Metestrus' and 'Diestrus', and 'Proestrus' differed significantly from 'Metestrus' and 'Diestrus' ($P < 0.05$; Chi-square test, followed by Chi-square or Fisher's test as 'post hoc' analysis).

1 – 'Proestrus'

Ovaries contained type 7 follicles, but no recent CLs (types Ia and Ib); type II and/or type III CLs were present in most cases. The uterus was dilated with fluid, and/or the vaginal epithelium was cornified, in approximately 50 % of these females; however, the vagina was closed in 4 out of 14 animals.

2 – 'Estrus'

The ovaries had large Graafian follicles (type 8) or recently ruptured, unluteinized ones. A cornified vaginal epithelium, a uterus dilated with fluid, and one or two sets of regressing CLs (type II and/or III) were found in most cases.

3 – 'Metestrus'

Ovaries contained newly formed CLs (type Ia); in 2 cases these contained trapped ova. Most ovaries also had one or two older generations of CLs (types II and/or III). The largest growing follicles varied from stages 5b to 7. The vaginal epithelium had always lost the cornified layer. The uterus was sometimes dilated with fluid.

4 – 'Diestrus'

Ovaries in which the largest follicle was type 5b or 6, and the most recent CLs were either type Ib (i.e., similar to Ia, but with no cavities nor 'channels') or type II. One or two older generations of CLs were also common. The vaginal epithelium was never cornified and sometimes was atrophic. The uterus was sometimes dilated with fluid.

5 – 'Anestrus'

The largest follicle was a type 5b (occasionally, type 6), and follicles in varying degrees of atresia were typical. The CLs were absent, or old (type III). The uteri were small and thread-like, with no intraluminal fluid. Vagina histology was very variable: the most common was a low squamous epithelium, infiltrated with leucocytes. When the vaginal orifice was closed (62.5 % of cases), the epithelium was atrophic and frequently covered with a layer of columnar mucous cells. In some cases with open vaginas, however, the epithelium was cornified. This can be correlated with the occasional days of cornification that interrupt the leucocytic smear pattern of these animals.

6 – 'Atretic cycles'

The largest growing follicles were type 5b or 6. CLs were absent or regressing (type II or III). In addition, there was a set of large atretic follicles, that appeared to have interrupted their growth simultaneously, contrasting with the asynchronous stages of atresia commonly found in anestrus ovaries. The incidence of type II and/or type III CLs (indicative of ovulation in the preceding cycle) did not differ from that in females undergoing ovulatory cycles, but was significantly higher than in anestrus females. The uteri were also significantly heavier than those of anestrus females, and were sometimes dilated with fluid. Vagina histology was as variable as in anestrus, and cornified epithelia were also found.

II - Patterns of changes in the vaginal smear

The vaginal smear of females that were not in anestrus at sacrifice (Fig. 1A) was characterized by the cyclic occurrence of waves of nucleated and cornified cells (modal length: 2 days), that were often accompanied by enlarged and swollen vaginal orifice. The onset of these epithelial waves was indicated by a decrease in the proportion of leucocytes and the appearance of cornified cells, sometimes preceded by small, nucleated epithelial cells (intensely stained with light green). Leucocytes disappeared from the smear only rarely, although they were definitely reduced in number during the epithelial waves. At the end of these waves, cornified cells disappeared, and a stage dominated by large, nucleated epithelial cells, sometimes preceded the ensuing series of predominantly leucocytic smears. Mucous smears occurred frequently during the leucocytic intervals between epithelial waves. The vaginal orifice narrowed during these intervals and sometimes closed completely.

Despite following the vaginal cycle before sacrificing animals, the vaginal smear frequently did not correlate closely with the status of the ovary at autopsy. For instance, proestrous ovaries sometimes coexisted with closed vaginas, or smears that varied from fully leucocytic to fully cornified ones, and fully cornified smears, typical of estrus, were

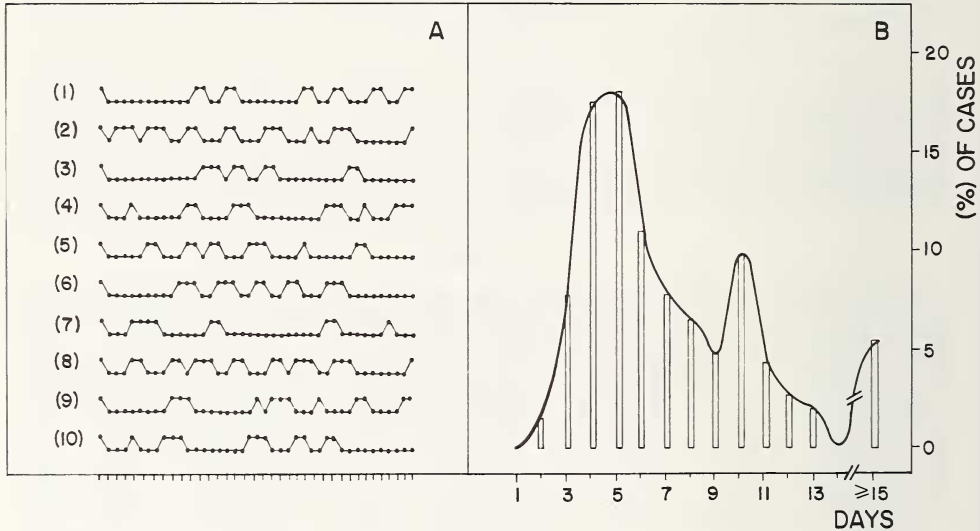


Fig. 1. Sequence of vaginal cycles in 10 selected cases that were observed during forty days (panel A); the upper points of each line on panel A indicate the days dominated by epithelial cells (either nucleated or cornified ones), while the lower points indicate the days of the diestrous intervals. Panel B shows a histogram of duration of vaginal cycles in 57 females that were not found in anestrus at sacrifice

sometimes found in anestrus animals. The disappearance of the cornified layer at metestrus, however, was clearly and reproducibly reflected in the smear and was the only vaginal event that could be reliably correlated with a change in the ovary.

A histogram of cycle lengths of females that were not in anestrus at sacrifice is shown in Figure 1B. The cycles were considered to begin when cornified cells disappeared from the smear, and to continue until the next disappearance of cornified cells: 182 cycles were recorded, from 57 females. Cycle length was very variable, with a mode of 5 days and a second frequency peak at 10 days.

On the other hand, females found in anestrus at sacrifice, were characterized by periods

of persistent leucocytic smears and/or closed vaginal orifices. In half of these, also, there was at least one episode of smears dominated by nucleated and/or cornified cells. However, these periods of epithelial cells were shorter (modal length: 1 day) than in non-anestrous females.

III – Incidence of the different phases

The frequency of the different ovarian stages was determined in 47 females that were not smeared, and were sacrificed on random days. Anestrus was frequent (38.3%), and the “atretic cycle” was infrequent (8.5%). Proestrus, estrus and metestrus showed about the same incidence as the atretic cycle (10.0, 8.5 and 10.6%, respectively), while diestrus was about twice as frequent (23.4%). Then, if we consider that cyclic animals showed a modal cycle length of 5 days, we can estimate the duration of proestrus, estrus and metestrus as one day each, and the duration of diestrus as two days.

IV – Plasma progesterone levels in the different ovarian states and in males

Plasma progesterone differed significantly ($P < 0.05$; Tukey test) in diestrous females whether their most recent CLs were type Ib or II. It was higher in mice bearing type Ib CLs, which were usually found (8 out of 9 cases) on the third day of leucocytic smears, i.e., approximately on the second day of diestrous (Table 3). Progesterone levels in type Ib diestrus were also higher ($P < 0.05$ or better; Tukey test) than in any other group except estrus. Conversely, plasma progesterone during estrus was also significantly higher ($P < 0.01$; Tukey test) than in any other group except type Ib diestrus. Males, and those females in anestrus, atretic cycles, proestrus, and metestrus had basal progesterone levels, and did not differ significantly between them nor with type II diestrus.

Discussion

The vaginal smear pattern of cyclic female corn mice is difficult to interpret if compared with the common laboratory species, partly because of the lack of a definite indication of proestrus. Indeed, the corn mouse does not show the surface layer of swollen cells that occurs at proestrus in the rat and other mammals (e.g. LONG and EVANS 1922), and that brings about the massive shedding of proestrous rounded cells in those species. Another complicating factor may be the contamination of smears with cells from preceding stages that may be retained in the double cervicovaginal folds that are typical of the corn mouse (CASTRO-VAZQUEZ et al. 1987).

It is then important to conclude that nothing can be said about the ovarian status of a living female, as in field studies, either from the vaginal smear or from the appearance of the external vaginal orifice on the day of capture. In the laboratory, however, where vaginal smears can be studied during subsequent days, total cycle length can be estimated as the distance between two disappearances of cornified cells, since the occurrence of newly formed CLs (type Ia) was reliably associated with that vaginal event. Under these

Table 3. Plasma progesterone concentration (nmol/l; mean \pm S.E.M.) in female Corn mice in different ovarian stages, and in males

| | Number of cases | Plasma progesterone (a) |
|-------------------|-----------------|-------------------------|
| Proestrus | 8 | 20.0 \pm 2.9 |
| Estrus | 10 | 49.9 \pm 8.9 |
| Metestrus | 15 | 19.7 \pm 1.9 |
| Diestrus, type Ib | 9 | 40.1 \pm 3.2 |
| Diestrus, type II | 11 | 21.0 \pm 2.9 |
| Atretic cycles | 5 | 12.4 \pm 1.6 |
| Anestrus | 21 | 16.5 \pm 1.3 |
| Males | 10 | 17.2 \pm 4.1 |

(a) Either ‘Estrus’ or ‘Diestrus, type Ib’ differed significantly from all other groups ($P < 0.05$; Tukey test). Other differences were not significant

conditions, the length of the cycle most frequently observed was 5 days, although somewhat longer cycles were also rather frequent, probably indicating the occurrence of short anestrus periods between the ovulatory cycles.

Short cycles as those described in the corn mouse have not been found outside the Superfamily Muroidea, but have been also described in many Muridae and Cricetidae, both from the Old World and from North and South America (ASDELL 1964; JUSTINES and JOHNSON 1970; CONAWAY 1954; DEWSBURY et al. 1977), which suggests that this may be an early acquisition within this Superfamily. This short cycle has been always observed in association with spontaneous ovulation, and with an induced luteal phase (type III cycle in the classification of CONAWAY 1971), and appears typical of short life-span, high reproduction prey species. It seems that the mechanism of ovulation in the corn mouse is basically spontaneous, since ovarian evidence of past or impending ovulation was observed in about 60% of females caged without males. We have not studied, however (and there are no reports, either) if it is possible to induce luteal activation by coital or cervicovaginal stimulation in the corn mouse. Therefore, we can only provisionally classify the corn mouse as bearing a type III cycle. A prolongation of luteal function after genital stimulation has been reported in some North American cricetids (DEWSBURY and ESTEP 1975; KENNEY et al. 1977), which are phylogenetically rather close to the South American ones.

Plasma progesterone in the corn mouse attains maximum levels during estrus and, after a nadir during metestrus, it peaks again in type Ib diestrus. A pattern of progesterone secretion that includes both a preovulatory (proestrus) and a postovulatory (diestrus) peak has been reported in many murid rodents, including the laboratory rat (BUTCHER et al. 1974; SMITH et al. 1975). It should be noted, however, that what we have designated here as 'estrus' is equivalent to what was called 'proestrus' in those studies. We preferred to designate this stage as 'estrus' in the corn mouse, since ovulation had already occurred in 4 out of 21 'estrous' animals that were sacrificed in the evening, and since ovulation only occurs about 10 hours later in the rat (EVERETT et al. 1949), i.e., in the early hours of the following day. However, both the estrous and the diestrus peaks of progesterone secretion are remarkably lower in the corn mouse than in the corresponding stages in the rat. The low level of circulating progesterone at estrus in the corn mouse may be correlated with the persistence of uterine fluid during metestrus and diestrus that occurs in some subjects, since progesterone secretion during the rat proestrus seems responsible for the rapid loss of uterine fluid that occurs in that species (BARNEA et al. 1968). Anovulating female corn mice, as well as cycling females in stages other than estrus and type Ib diestrus, had low progesterone levels similar to those found in males. The occurrence of basal progesterone levels in late diestrus (type II) indicates that the function of the corpora lutea formed at ovulation is ephemeral, and further suggests that some form of luteal activation (induced luteal phase) should exist to render implantation possible.

The high incidence of anestrus in laboratory-reared corn mice may be explained, in principle, as an effect of (1) some dietary deficiency (for instance, 6-methoxybenzoxazolinone: BERGER et al. 1981; SANDERS et al. 1981; SCHADLER et al. 1988); (2) grouping (which may be act either through a pheromonal effect: CHAMPLIN 1977; or through establishing social ranks within the group: BUJALSKA 1973), and (3) prevention of male exposure (MONTORO et al. 1987). No effort was made here to determining the cause of anestrus in our laboratory conditions. It is clear, however, that the high incidence of anestrus is an effect of captivity, since we found (MILLS et al. 1992) that all feral adult females are reproductively active when captured during the breeding season.

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Zusammenfassung

Beziehungen zwischen Genitaltraktmorphologie und Plasmaprogesteronspiegel während des ovariellen Zyklus von Maismäusen (Calomys musculinus)

Beschrieben wird die Beziehung zwischen der Morphologie des Genitaltrakts und dem Progesteronspiegel im Plasma der Maismaus (*Calomys musculinus*). Morphologische Charakteristika des Ovars, des Uterus und der Vagina wurden zur Definition der Zyklusphasen und einiger anovulatorischer Zustände benutzt. Die Befunde wurden mit Veränderungen in Vaginalabstrichen in Beziehung gebracht sowie mit der Progesteronkonzentration im Plasma (gemessen mit Hilfe eines Radioimmunoassay). Bei Weibchen, die ohne Männchen gehalten wurden, traten Östrus und Ovulation zyklisch auf. Sowohl während wie auch nach der Ovulation zeigte die Progesteronkonzentration im Plasma Spitzenwerte. Veränderungen in den Vaginalabstrichen zeigten nur geringe Beziehungen zu Veränderungen im Ovar. Die Ausbildung frischer Gelbkörper war allerdings mit dem plötzlichen Verschwinden von Zellen des Stratum corneum aus dem Abstrich verbunden. In der Mehrzahl der Fälle betrug die Länge des Zyklus fünf Tage.

Der Ovarialzyklus der untersuchten Art wird in Anlehnung an CONAWAY (1971) vorläufig dem Typ III zugeordnet, welcher durch spontanen Eisprung und induzierte Lutealphase charakterisiert ist.

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